

ZED1-related kinases (ZRKs) from *Aquilegia coerulea* and *Cinnamomum micranthum* do not trigger autoactive cell death in *Nicotiana benthamiana*

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ZED1-related kinases (ZRKs) are receptor-like cytoplasmic kinases (RLCK) that mediate pathogen effector recognition and hypersensitive cell death response via the intracellular immune receptor HOPZ-ACTIVATED RESISTANCE1 (ZAR1). To investigate the functional connection between ZAR1 and ZRK orthologs across flowering plant species, Adachi et al. (2022) cloned *ZAR1* and *ZRK* homologs from *Cinnamomum micranthum* and *Aquilegia coerulea*. Here, we performed a transient gene expression screening to determine whether expression of wild-type ZRKs causes autoactive cell death in the model plant *Nicotiana benthamiana*. In total, we screened 13 and five *ZRK* genes from *C. micranthum* and *A. coerulea*, respectively. None of the tested ZRKs triggered macroscopic cell death response when expressed in *N. benthamiana* leaves. This indicates that *C. micranthum* and *A. coerulea* ZRKs are not autoactive and do not trigger cell death in the absence of their partner ZAR1.

Introduction and result

NLR (Nucleotide-binding leucine-rich repeat) proteins are intracellular immune receptors that play a pivotal role in recognizing pathogen-secreted effectors and activating robust immune responses, including the hypersensitive cell death response (Jones et al., 2016). Plant NLRs typically consist of a central NB-ARC (nucleotide-binding domain shared with APAF-1, various R-proteins and CED-4) domain, a C-terminal leucine-rich repeat (LRR) domain, and an N-terminal domain, which includes Rx-type coiled-coil (CC) domain, RPW8-type CC (CC_R) domain or Toll/Interleukin-1 receptor (TIR) domain (Kourelis et al., 2021). As a consequence of their coevolution with pathogens, NLR genes underwent lineage-specific expansions in plant genomes and are known as the most diversified gene family in plants (Van de Weyer et al., 2019).

Adachi et al. (2022) conducted phylogenomics analyses of plant CC-NLRs and revealed that HOPZ-ACTIVATED RESISTANCE1 (ZAR1) is the most conserved CC-NLR gene across flowering plant species. ZAR1 orthologs were identified from 88 species including monocot, magnoliid and eudicot, suggesting the origin of ZAR1 in flowering plant lineages traces back to ~220 to 150 million years ago (the Jurassic period). ZAR1 indirectly recognizes bacterial pathogen effectors through its partner receptor-like cytoplasmic kinases (RLCKs) (Wang et al., 2015; Wang et al., 2019a; Wang et al., 2019b; Hu et al., 2020). The partner RLCKs are also known as ZED1-related kinase (ZRK) proteins mainly from RLCK XII-2 subfamily (Lewis et al., 2013). ZRK shows consistent gene distribution with ZAR1 across flowering plant species (Gong et al., 2022), suggesting that ZAR1 and ZRK have coevolved to recognize pathogen effectors since the Jurassic period.

To functionally validate ZAR1 and ZRK from distantly related plant species, Adachi et al. (2022) cloned ZAR1 and ZRK genes from Magnoliidae *Cinnamomum micranthum* (Syn. *C. kanehirae*, stout camphor) and Ranunculales *Aquilegia coerulea* (columbine). Here, we screened stout camphor ZRKs (CmZRK) and columbine ZRKs (AcZRK) for autoactive cell death response in *Nicotiana benthamiana* leaves. None of 13 CmZRKs (CmZRK2, CmZRK3, CmZRK4, CmZRK5, CmZRK6, CmZRK7, CmZRK8, CmZRK9, CmZRK10, CmZRK11, CmZRK12, CmZRK13, CmZRK15) and five AcZRKs (AcZRK1, AcZRK2, AcZRK3, AcZRK4, AcZRK5) (Adachi et al., 2022), triggered macroscopic cell death response when expressed in *N. benthamiana* leaves (Figure 1). These results indicate that CmZRKs and AcZRKs do not have autoactivity in *N. benthamiana* and, therefore, can be used for co-expression assays with ZAR1 NLR proteins to study their function in immunity.

Materials and Methods

Plant growth condition

Wild-type *Nicotiana benthamiana* plants were grown in a controlled growth chamber with temperature 22-25 °C, humidity 45-65% and 16/8 hr light/dark cycle.

Plasmid information

ZAR1 and ZRK genes used in this study were previously cloned into the binary vector pICH86988 (Harant et al., 2022; Adachi et al., 2022). Detailed plasmid information is described in Table 1.

Agrobacterium-mediated transient gene expression

Transient expression of ZAR1 and ZRK in *N. benthamiana* leaves were performed by agroinfiltration according to methods described by Bos et al. (2006). Leaves of four-weeks old *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* GV3101 pMP90 strains carrying the binary expression plasmids. *A. tumefaciens* suspensions in infiltration buffer (10 mM MES, 10 mM MgCl₂, and 150 µM acetosyringone, pH5.6) were adjusted to OD₆₀₀ = 0.25. Macroscopic cell death response was observed four days after agroinfiltration.

Accession numbers

Each sequence information used in this study can be found in the GenBank database with the following accession numbers: NbZAR1 (AXY05280.1), CmZAR1 (RWR84015.1), AcZAR1 (PIA46011.1), CmZRK2 (RWR79813.1), CmZRK3 (RWR79826.1), CmZRK4 (RWR79825.1), CmZRK5 (RWR79822.1), CmZRK6 (RWR79846.1), CmZRK7 (RWR79827.1), CmZRK8 (RWR79848.1), CmZRK9 (RWR79816.1), CmZRK10 (RWR79844.1), CmZRK11 (RWR79823.1), CmZRK12 (RWR79850.1), CmZRK13 (RWR81459.1), CmZRK15 (RWR93701.1), AcZRK1 (PIA24723.1), AcZRK2 (PIA41834.1), AcZRK3 (PIA41836.1), AcZRK4 (PIA41835.1), AcZRK5 (PIA41837.1).

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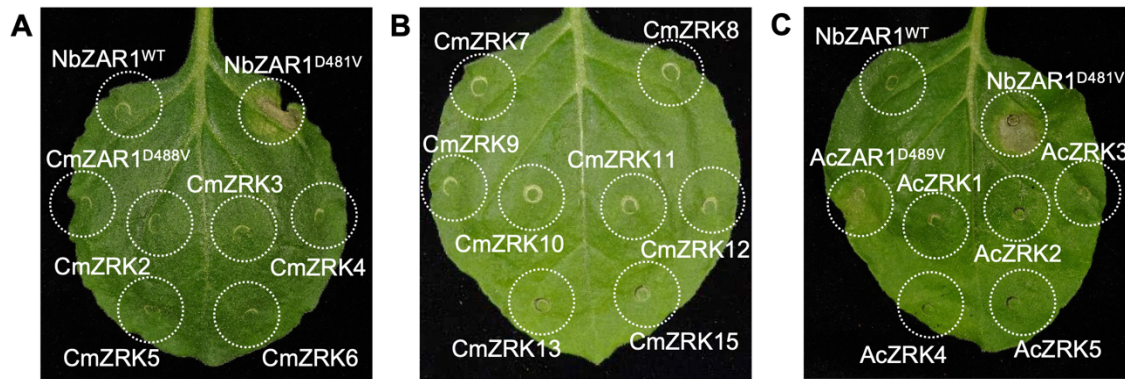


Figure 1. *Cinnamomum micranthum* and *Aquilegia coerulea* ZRKs do not induce macroscopic cell death response in *Nicotiana benthamiana* leaves. (A and B) Wild-type *C. micranthum* ZRKs (CmZRKs) were expressed in *N. benthamiana* leaves by agroinfiltration. Wild-type *N. benthamiana* ZAR1 (NbZAR1^{WT}), MHD motif mutants of NbZAR1 (NbZAR1^{D481V}) and CmZAR1 (CmZAR1^{D488V}) were used as controls. (C) *A. coerulea* ZRKs (AcZRKs) and MHD motif mutant of AcZAR1 (AcZAR1^{D489V}) were expressed in *N. benthamiana* leaves by agroinfiltration. Photographs were taken at four days after agroinfiltration and describe representative data from at least ten replicates. NbZAR1^{D481V} and AcZAR1^{D489V} caused autoactive macroscopic cell death response as reported in Adachi et al. 2022.

Table 1. Binary vector plasmids used in this study.

Insert gene ID	Plasmid	Antibiotic	Plasmid ID	Publication
AXY05280.1	pICH86988-NbZAR1	Kan	pZA21	Harant et al., 2022
MHD mutant of AXY05280.1	pICH86988-NbZAR1_D481V	Kan	pZA22	Harant et al., 2022
MHD mutant of PIA46011.1	pICH86988-AcZAR1_D489V	Kan	pJK-B2-679	Adachi et al., 2022
PIA24723.1	pICH86988-AcZRK1	Kan	pJK-B2-680	Adachi et al., 2022
PIA41834.1	pICH86988-AcZRK2	Kan	pJK-B2-681	Adachi et al., 2022
PIA41836.1	pICH86988-AcZRK3	Kan	pJK-B2-682	Adachi et al., 2022
PIA41835.1	pICH86988-AcZRK4	Kan	pJK-B2-683	Adachi et al., 2022
PIA41837.1	pICH86988-AcZRK5	Kan	pJK-B2-684	Adachi et al., 2022
MHD mutant of RWR84015.1	pICH86988-CmZAR1_D488V	Kan	pJK-B2-691	Adachi et al., 2022
RWR79813.1	pICH86988-CmZRK2	Kan	pJK-B2-693	Adachi et al., 2022
RWR79826.1	pICH86988-CmZRK3	Kan	pJK-B2-694	Adachi et al., 2022
RWR79825.1	pICH86988-CmZRK4	Kan	pJK-B2-695	Adachi et al., 2022
RWR79822.1	pICH86988-CmZRK5	Kan	pJK-B2-696	Adachi et al., 2022
RWR79846.1	pICH86988-CmZRK6	Kan	pJK-B2-697	Adachi et al., 2022
RWR79827.1	pICH86988-CmZRK7	Kan	pJK-B2-698	Adachi et al., 2022
RWR79848.1	pICH86988-CmZRK8	Kan	pJK-B2-699	Adachi et al., 2022
RWR79816.1	pICH86988-CmZRK9	Kan	pJK-B2-700	Adachi et al., 2022
RWR79844.1	pICH86988-CmZRK10	Kan	pJK-B2-701	Adachi et al., 2022
RWR79823.1	pICH86988-CmZRK11	Kan	pJK-B2-702	Adachi et al., 2022
RWR79850.1	pICH86988-CmZRK12	Kan	pJK-B2-703	Adachi et al., 2022
RWR81459.1	pICH86988-CmZRK13	Kan	pJK-B2-704	Adachi et al., 2022
RWR93701.1	pICH86988-CmZRK15	Kan	pJK-B2-706	Adachi et al., 2022